Hexafluoroisopropanol and Acid Destabilized Forms of Apomyoglobin Exhibit Structural Differences†

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Received July 1, 2002; Revised Manuscript Received September 25, 2002

ABSTRACT: The conformational properties of partially folded states of apomyoglobin have been investigated using an integrated approach based on fluorescence spectroscopy and hydrogen/deuterium exchange followed by mass spectrometry. The examined states were those obtained: (i) by adding 4% v/v hexafluoroisopropanol to native myoglobin, HFIP-MG_N; (ii) by adding 4% v/v hexafluoroisopropanol to acid unfolded myoglobin, HFIP-MG_U; (iii) at pH 3.8, I-1 state; and (iv) at pH 2.0−0.2 M NaCl, A state. Proteolytic digestion of the hydrogen/deuterium exchanged proteins showed that, in I-1 state, the helices C, D, E, and F incorporate more deuterium, whereas in HFIP-MG_N the exchange rate is similar for all protein regions. These results suggest that I-1 contains the ABGH domain in a native-like organization, whereas HFIP-MG_N loses a large number of tertiary interactions, thus acquiring a more flexible structure. The fluorescence data are consistent with the above picture. In fact, the tryptophan/ANS energy transfer is much less efficient for the ANS−HFIP-MG_N complex than for the other complexes, thus suggesting that the distances between the fluorophores might be increased. Moreover, fluorescence polarization measurements indicated that the rotational motion of HFIP-MG_N occurs on a longer time scale than the other partially folded states, thus suggesting that the volume of this state could be larger. The overall results indicate that addition of hexafluoroisopropanol to native myoglobin results in the formation of a true molten globule where tertiary interactions are reduced, while the secondary structure and the globular compactness are conserved.

Myoglobin is a small globular protein in which eight α helices form a hydrophobic cage where the prosthetic group can bind. The eight helical segments are usually indicated with capital letters from A to H. In the holoprotein, the heme closely interacts with helices E and F. At low ionic strength, the acid induced unfolding of myoglobin consists of a four-state process: Mb ↔ ApoMb + H ↔ I-1↔ U, where H denotes the hemin free in solution after dissociation, and I-1 denotes a partially folded intermediate appearing on the denaturation pathway (*1*−*5*). The stability of myoglobin is primarily determined by the rate of hemin dissociation. As the pH is reduced, dissociation occurs, and the globin portion is rapidly converted in the I-1 state. This is an intermediate, partially structured state, characterized by a fully disorganized hemin site (*1*, *6*, *7*). On the basis of CD and NMR studies, it has been suggested that the intermediate form I-1 appearing near pH 4.0 is composed of a structured subdomain, formed namely by the helices A, G, and H, whereas the remainder of the molecule is essentially disordered (*8*−*12*). The I-1 state is strongly stabilized by salt addition. The salt stabilization makes the acid induced unfolding at high ionic strength similar to a two-state process in which the two in-equilibrium forms are the native state and the partially structured one (*13*, *14*). The structural features of I-1 state are very similar to those found for the 100 ms intermediate observed in the refolding pathway of apomyoglobin (*12*, *15*, *16*). A second, slightly more structured state, having the B helix in a structured form (i.e., I-2 state), has been detected by adding trichloroacetic acid to I-1 (*17*). More recently, a destabilized form of the protein has been observed at pH 3.0 and low ionic strength. This form has been referred to as E state because it appears to be substantially unfolded and extended (*18*). Nevertheless, although less helical, it still seems to contain a distinctive organized core composed of 20−40 residues at the intersection of the A helix with the G and H helices and the G−H hairpin turn (*18*, *19*). Thus, the formation of the AGH core appears to be a critical step in the folding of apomyoglobin.

Partially folded states are also induced by moderate concentrations of alcohol. Alcohol destabilizes hydrophobic cores of protein because of its nonpolar character, while it enhances secondary structure formation by minimizing exposure of the peptide backbone (*20*−*22*). These effects destabilize the native state of the protein and induce a helical denatured conformation (*23*−*29*). The thermodynamic force inducing secondary structure of the protein may also determine a collapse of a denatured protein chain by enhancing intrachain hydrogen bonding, and thus some alcohols having strong helical induction may stabilize a

† This work has been supported by PRIN 2000 Partly Folded States of Proteins: Analysis by an Integrated Approach of Biochemical and Biophysical Methods from the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST−Rome).

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10.1021/bi020447f CCC: $25.00
Published on Web 12/17/2002

compact state by overcoming the expanding force exerted by the nonpolar character of alcohol. 1,1,1,3,3,3-hexafluoroisopropanol (HFIP)[1] has these features (*30*−*31*). Cort and Andersen have characterized the apomyoglobin molten globule states obtained by adding 4% v/v HFIP either to native or to acid unfolded protein (*32*). They found that the fluoroalcohol disrupts the tertiary structure of native myoglobin, while the secondary structure is largely conserved. By contrast, adding the fluoroalcohol to the acid unfolded protein produces secondary structure but no tertiary interactions.

Major advances in protein conformation studies were achieved when integrated strategies combined experimental data from different techniques. Here, we perform a conformational characterization of molten globules of apomyoglobin obtained under different experimental conditions by examining the fluorescence emission of a fluorescent probe bound to the protein and using hydrogen/deuterium exchange (HDX) experiments followed by mass spectrometry.

Naphthalene-based fluorescent probes such as 1-anilino-8-naphthalene sulfonic acid (ANS) have commonly been used to examine the conformational properties of protein accessible hydrophobic pockets (*33*−*37*). Recent investigations have shown the ability of ANS molecules to bind to non-native structural states of proteins with a pronounced secondary structure and compactness but without a tightly packed tertiary structure (i.e., molten globule state (*38*−*40*)). Currently, ANS represents a standard probe for investigating the population of compact partially folded states of proteins (*41*−*46*).

HDX at labile positions (i.e., the peptide amide linkages) mainly depends on protein conformation. In fact, hydrogen atoms are readily exchanged with deuterium unless the conformation of the protein prevents solvent accessibility and/or the amide hydrogen atoms are involved in intramolecular interactions. HDX experiments can then be used to evaluate conformational differences between proteins or even intermediate states in different experimental conditions (*47*−*50*). Although hydrogen exchange has most often been detected by NMR, monitoring by mass spectrometry (MS) has become increasingly common (*51*−*55*). As deuterium atoms replace protons during the hydrogen exchange period, the mass of the protein increases. The extent as well as the rate of exchange can then be determined by monitoring the change in protein mass value.

The results reported in this paper indicate that the addition of 4% v/v HFIP to native myoglobin induces structural changes different from those observed for apomyoglobin at low pH or induced by adding salt or fluoroalcohol to acid unfolded myoglobin.

EXPERIMENTAL PROCEDURES

Horse myoglobin was purchased from Sigma; the protein was used after a further purification step performed by fast liquid chromatography using a Superdex 75 column (16 mm × 60 cm) equilibrated with 0.01 M phosphate (pH 7.0). Myoglobin concentration was determined spectrophotometrically in the Soret region using molar extinction coefficient $\epsilon_{409} = 157\,000\ M^{-1}\ cm^{-1}$ (*56*). The heme was removed from myoglobin by a 2-butanone extraction procedure (*57*). The heme contamination of the apoprotein was assessed spectrophotometrically; no significant absorption was observed in the Soret region. The concentration of apomyoglobin was determined by absorption at 280 nm using the following molar extinction coefficient $\epsilon_{280} = 13\,500\ M^{-1}\ cm^{-1}$ calculated from the tryptophan and tyrosine content (*58*). HFIP ($CF_3CHOHCF_3$) was purchased from Sigma and was distilled prior to use. 1-anilino-8-naphthalene-sulfonate (ANS) was purchased from Molecular Probes. The ANS concentration was determined spectrophotometrically using $5 \times 10^3\ M^{-1}\ cm^{-1}$ as the molar absorption coefficient at 350 nm (*59*).

Fluorescence Measurements. Fluorescence spectra were measured by a LS50 fluorometer (Perkin-Elmer) using a quartz cell with a light path of 10 mm. Bandwidths for excitation/emission light were 3.5/3.0 nm. The temperature of sample solutions was maintained at 25 °C by a thermostatically controlled water bath. Titrations with ANS were made manually by adding small aliquots of concentrated ANS to protein solution. Dilutions on ANS never exceeded 5% of the starting volume. The protein concentration was 20 μM. The baselines were obtained using the same concentration of free fluorophore at each experimental condition. In all cases, the fluorescence intensity of the free fluorophore was much smaller than that of the ANS−protein complex. Measurements were monitored through time until an apparent equilibrium was reached. The fluorescence intensity decreased linearly with sample concentration from 20 to 0.8 μM indicating that the intermediate states were not aggregated.

HDX. Isotopic exchange experiments were performed on native apomyoglobin and on partly folded intermediates. These were previously obtained by incubating myoglobin in suitable conditions (i.e., 10 mM ammonium acetate (pH 4.2) containing 4% (v/v) HFIP-MG_N and 10 mM ammonium acetate, pH 3.8 (I-1)). Deuterium exchange reactions were initiated by 10-fold dilution of protein solutions with analogous buffer prepared in D_2O. The exchange reaction was allowed to proceed for a length of time ranging from 10 s to 60 min, and at each time, 0.5 nmol of protein was removed from the labeling solution and rapidly injected into a 30 × 0.46 mm i.d. perfusion column (POROS 10 R2 media, Applied Biosystems) coupled to an API-100 single quadrupole mass spectrometer (Applied Biosystems). The protein was eluted at a flow rate of 1 mL/min with a gradient of 25−95% acetonitrile in TFA 0.1% in 1.0 min. The HPLC step was performed with cold protiated solvents, thereby reducing the back-exchange kinetics and removing deuterium from side chains and amino/carboxy termini that exchange much faster than amide linkages (*60*, *61*). The increase in molecular mass of the protein sample then constituted a direct measurement of deuterium incorporation at peptide amide linkages. Data were acquired and elaborated using the Biomultiviewer (Applied Biosystems) program. Duplicate analyses were performed for each time point. Experimental data were fitted to a double exponential model using the following equation:

$$D = N_{fast}(1 - e^{-k_{fast}t}) + N_{slow}(1 - e^{-k_{slow}t})$$

[1] Abbreviations: HFIP, 1,1,1,3,3,3-hexafluoroisopropanol; ANS, 1-anilino-8-naphthalene-sulfonate; HDX, hydrogen/deuterium exchange; MS, mass spectrometry; LC/MS, liquid chromatography/mass spectrometry; ES, electrospray; ApoMb, apomyoglobin.

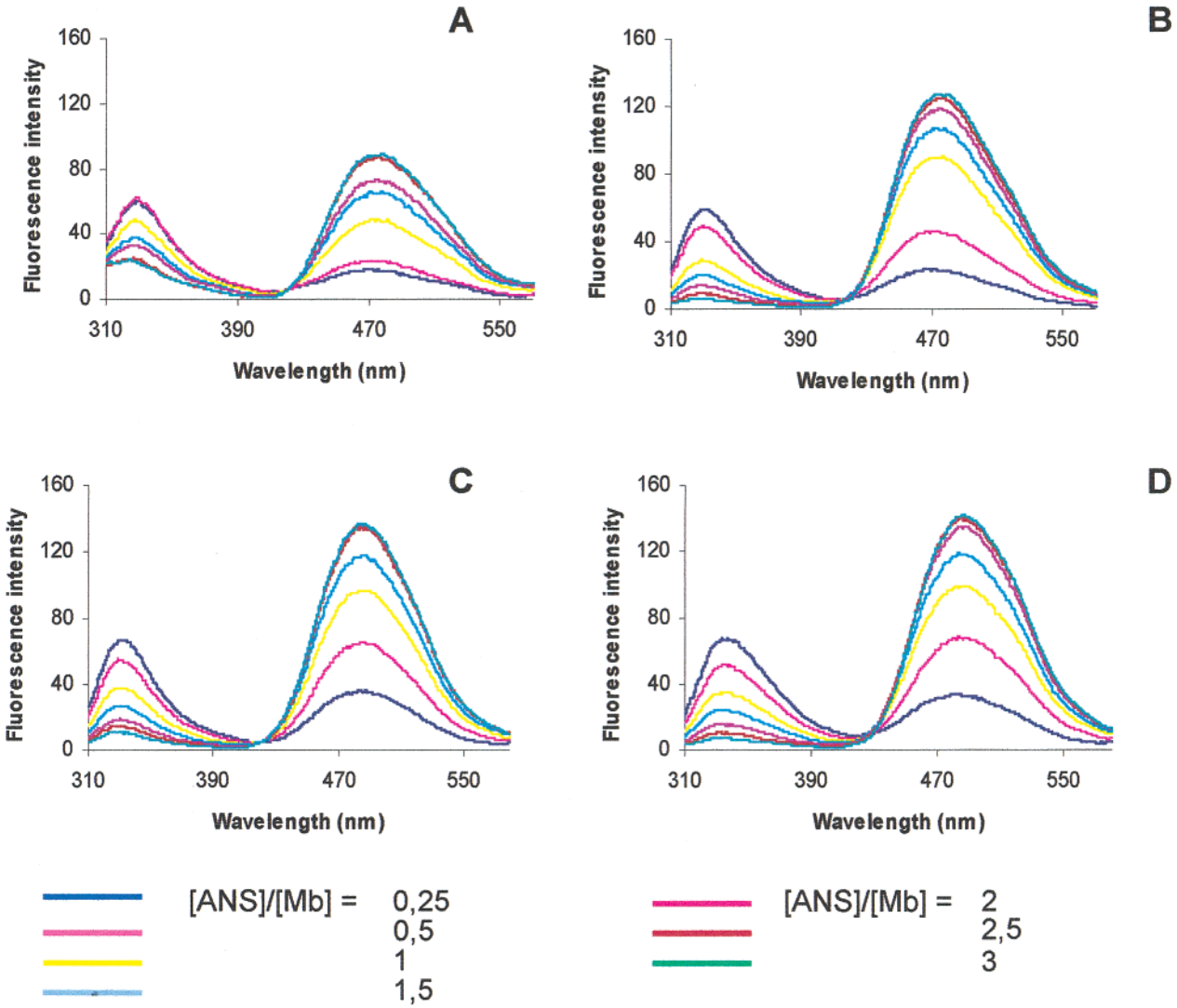

FIGURE 1: Fluorescence emission spectra of the complexes formed by ANS with partially folded states of apomyoglobin: HFIP-MG_N (A), I-1 state (B), A-state (C), and HFIP-MG_U (D). Protein concentrations were 20 μM. All solutions contain 0.01 M phosphate. Excitation was at 295 nm. Temperature was at 25 °C.

where D is the total number of deuterons at time, t, N_{fast} is the number of deuterons exchanging at the fast rate, k_{fast}, and N_{slow} is the number of deuterons exchanging at the slow rate, k_{slow}. Data were analyzed using KaleidaGraph 3.0 (Synergy Software, Inc.).

Proteolytic digestion of deuterated protein samples was performed on native apomyoglobin and on the two intermediates HFIP-MG_N and I-1 to obtain information on the different exposure of individual protein regions. Each species was allowed to exchange for 60 min as described above. Before analysis, 1 nmol of each sample was twice diluted with a 50 mM phosphate buffer (pH 2.5) containing 34 μg of pepsin and incubated for 5 min at 0 °C. Resulting peptides were fractionated by HPLC on the reverse-phase perfusion column using a 10−35% acetonitrile−water gradient in 8 min, as described for intact protein analysis. Mass spectrometry data were obtained and elaborated as previously described. All the exchange experiments were performed in triplicate.

RESULTS

ANS Fluorescence Studies. ANS is essentially nonfluorescent in aqueous solution and becomes strongly fluorescent in an apolar environment (*34*). The binding of the ANS to the molten globule state of apomyoglobin reduces the tryptophanyl fluorescence emission and results in the appearance of the characteristic emission of the fluorophore in apolar environment. Figure 1 shows the effect of increasing ANS concentration on the emission spectrum of the molten globule state formed by apomyoglobin under different experimental conditions. In particular, we examined the partially folded states formed in the following experimental conditions: (i) pH 4.2 in 4% v/v HFIP, referred to as HFIP-MG_N; (ii) pH 2.0 in 4% v/v HFIP, referred to as HFIP-MG_U; (iii) pH 3.8 in the presence of very low salt concentration, referred to as I-1 state; (iv) pH 2.0 in 0.2 M NaCl, referred to as the acidic compact state or A state. All the examined protein states retain some secondary structure as documented by far ultraviolet CD studies (*5*, *8*, *32*, *39*). The emission spectra reported in Figure 1, obtained with 295 nm excitation, show two emission bands: the first band at 330 nm is characteristic of tryptophanyl residues in a rather hydrophobic environment, and the second one at 475/480 nm is typical of ANS bound to partially folded apomyoglobin (*7*, *39*). As shown, the intensity of tryptophanyl emission is quenched on increasing ANS concentration, whereas the intensity of

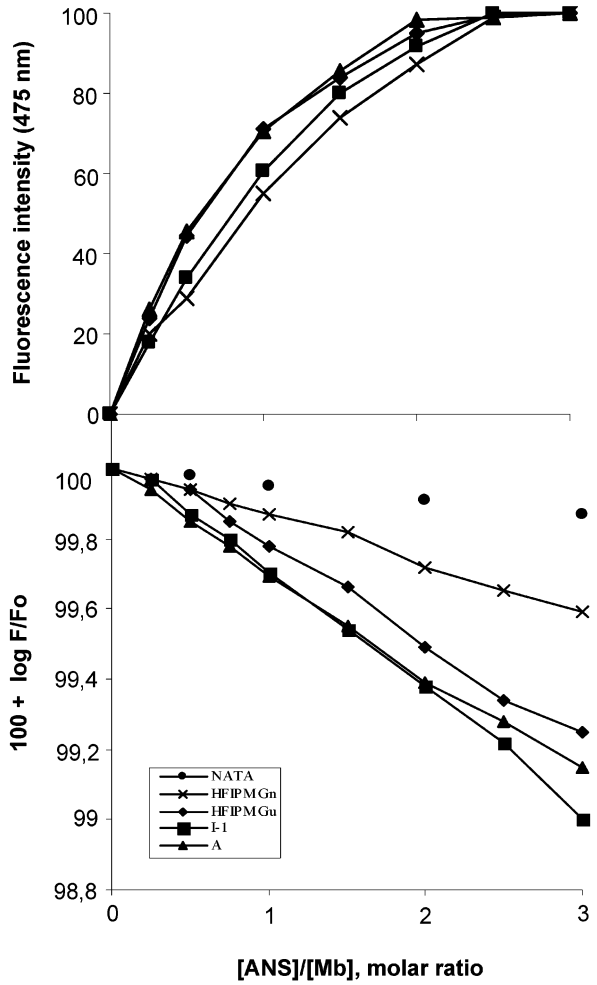

FIGURE 2: Effect of ANS increasing concentration on normalized fluorescence emission intensity at 475 nm (upper panel) and tryptophanyl emission intensity of partially folded apomyoglobin states (lower panel). Tryptophanyl fluorescence variations are expressed as [log F/F_0] + 100. The excitation wavelength was at 295 nm, and fluorescence intensity was monitored at 335 nm for partially folded apomyoglobin states and at 356 nm for *N*-acetyltryptophanamide, NATA. The other experimental conditions are those reported in Figure 1.

ANS emission increases. The ANS fluorescence increase reaches a plateau at [ANS]/[apoMb] molar ratios similar for all examined intermediate species (upper panel of Figure 2), thus suggesting that the affinity of the dye for the protein is essentially the same. The lower part of Figure 2 shows the effect of increasing ANS concentration on the emission intensity of tryptophanyl fluorescence. Linear plots are obtained by plotting the logarithmic values of tryptophanyl fluorescence decrease (F/F_0) versus the concentration of ANS. For comparison, the ANS concentration dependent quenching of the monomeric tryptophanyl residue (i.e., *N*-acetyltryptophanylamide (NATA)) is also shown. The fluorescence variations of both the extrinsic and intrinsic fluorophore can be taken as indicators of an energy transfer from tryptophan residues to the ANS fluorophore via the Förster mechanism. However, in the case of the complex formed by ANS with the molten globule state obtained by adding 4% v/v HFIP to native myoglobin (HFIP-MG_N), the fluorescence changes are much less pronounced than in the other examined cases. The ratios between ANS and tryptophanyl emission intensity taken at saturation are 4.0, 21.3, 22.5, and 20.5 for HFIP-MG_N, HFIP-MG_U, I-1, and A states, respectively. This result indicates that the energy transfer from tryptophanyl residues to ANS is more efficient in the ANS complexes formed by HFIP-MG_U, I-1, and A state.

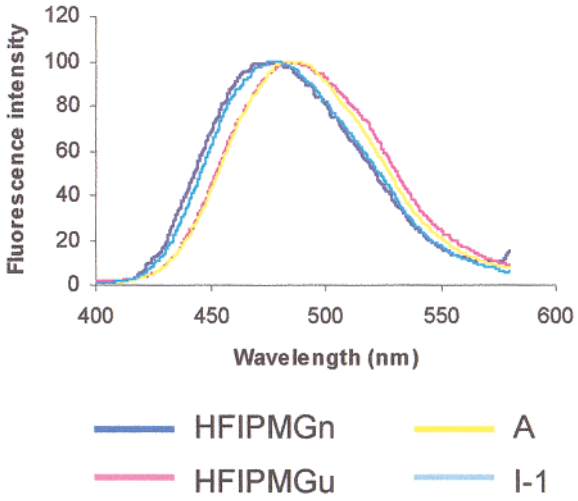

FIGURE 3: Normalized emission spectra of ANS−partially folded apomyoglobin complexes. Experimental conditions are those indicated in Figure 1.

Figure 3 shows the normalized emission spectra of ANS bound to molten globules of apomyoglobin. The emission maximum of the complexes formed at pH 2.0 (i.e., HFIP-MG_U and A state) is red-shifted with respect to that of the complexes obtained at pH 3.6−4.2. This can be due to pH-dependent charge effects in the proximity of the bound fluorophore.

ANS fluorescence polarization measurements at different [ANS]/[ApoMb] molar ratios were performed on HFIP-MG_N and I-1 states. The values were constant over the explored range of [ANS]/[ApoMb] molar ratios (from 0.75 to 4) and were 0.18 and 0.11 for HFIP-MG_N and I-1, respectively. The constancy of polarization suggests that no intramolecular transfer from ANS to ANS occurs, thus suggesting that one single fluorophore molecule is bound to apomyoglobin molten globules. Similar results were obtained for the other intermediate states. The observation that the degree of fluorescence polarization of HFIP-MG_N is significantly higher indicates that the rotational motion occurs on a longer time scale than the other states. If the fluorescent probe is supposed to be rigidly attached to the macromolecule, a polarization increase could be produced by a volume increase of the complex.

HDX Experiments. Conformational analysis of HFIP-MG_N and I-1 species were performed by HDX experiments followed by ES-MS. Analogous experiments were also carried out on native apomyoglobin for comparison and are in agreement with previously reported data (*62*). Protein solutions (20 μM) were diluted 10-fold by adding the appropriate D_2O buffers, and deuterium incorporation was monitored by sampling the incubation mixture at different time intervals followed by cold acid quenching and fast LC/MS analysis. The single envelope of isotope peaks observed in the mass spectra of the three species indicated a EX2 kinetics of HDX (*60*, *61*), confirming that these species are homogeneous and stable intermediates.

Figure 4 shows the number of amide protons exchanged by HFIP-MG_N, I-1, and native apomyoglobin as a function

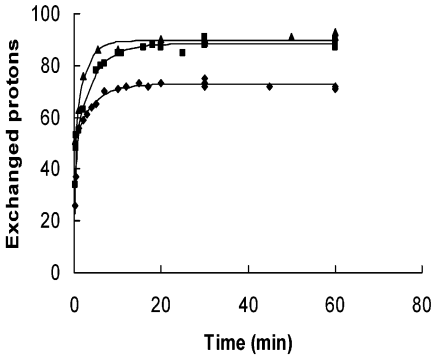

FIGURE 4: Number of exchanged protons in apomyoglobin (◆), HFIP-MG_N (■), and I-1 state (▲) was calculated by the increase in the molecular mass of the two proteins and is reported as a function of the exchange time. Continuous lines represent the best fit obtained using the biexponential equation reported in Experimental Procedures.

of time. Following 60 min of reaction, a total of 90 ± 2 hydrogen atoms were replaced with deuterium in both the intermediate states, whereas the native protein incorporated only 71 ± 1 deuterium atoms. The greater number of exchanged protons indicated that HFIP-MG_N and I-1 display less structured conformations than the native protein. This is consistent with a partial unfolding of the protein structure when passing from the native to the intermediate species. However, since about 38% of the amide protons in the intermediate forms are still shielded from the solvent, these species still retain a significant amount of secondary and/or tertiary structure.

Moreover, both HFIP-MG_N and I-1 intermediates underwent isotopic exchange with faster kinetics than native apomyoglobin, suggesting a higher degree of structural flexibility. The kinetic constants, k_{fast} and k_{slow}, obtained by fitting the experimental data with a double exponential model, were 20.7 ± 3.2 and 0.46 ± 0.03 for I-1 and 7.4 ± 0.8 and 0.25 ± 0.02 for HFIP-MG_N, respectively. Thus, I-1 showed faster kinetics than HFIP-MG_N although, at long reaction time, both intermediates exchanged essentially the same number of amide hydrogen atoms. This result is even more significant considering that the HDX experiments on the I-1 species were performed at a lower pH value, where the intrinsic amide exchange rate is sensibly lower (*60*). This observation suggests that the two intermediates possess a distinguishably different conformation.

The differences in conformational states for apomyoglobin under non-native conditions were further investigated by employing a strategy that combines isotopic exchange and proteolytic digestion. HFIP-MG_N and I-1 were allowed to exchange for 60 min and, then, incubated with pepsin in conditions that minimized isotopic back exchange of amide protons. The resulting peptide mixtures were directly analyzed by fast LC/MS technique. Analogous experiments were carried out on both deuterated and native protein as a reference. Peptide fragments were identified by their unique mass values by comparing them with the fragments released from the proteolysis of nondeuterated apomyoglobin. Determination of the molecular mass of deuterated fragments directly indicated the number of exchanged protons for each individual peptide.

Figure 5 shows the percentage of exchanged hydrogen atoms (with respect to the total number of amide protons)

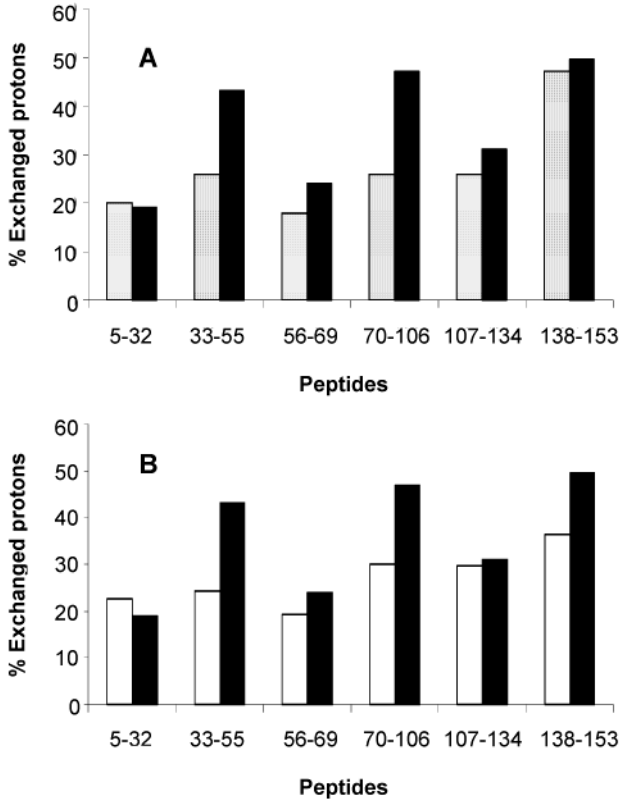

FIGURE 5: Percentage of exchanged protons in peptides generated by peptic digestion following 60 min incubation in buffered D_2O. (A) Apomyoglobin (gray bars) and I-1 state (■); (B) HFIP-MG_N (□) and I-1 state (■).

for individual fragments generated by peptic digestion of native apomyoglobin and the different intermediates. Since pepsin is nonspecific, a number of different fragments corresponding to the same protein regions was generated during proteolysis. The nonredundant series of the shortest peptides covering the entire myoglobin sequence was then considered in the analysis.

Figure 5A shows the differences in the HDX between native protein and I-1 state. As expected, the intermediate species displayed a slightly higher flexibility along the entire protein structure; however, two fragments, 33−55 and 70−106, corresponding to the helices C, D, E, and F showed a particularly large increase in deuterium incorporation. This result confirms previous CD and NMR findings indicating the occurrence in I-1 of a structured portion comprising helices A, G, and H with the remainder of the molecule being essentially unordered (*5*, *9*, *11*).

When the amide protons exchanged by the two intermediates were compared (Figure 5B), I-1 clearly showed a higher degree of deuterium incorporation selectively localized within three well-defined fragments (i.e., peptides 33−55, 70−106, and 138−153 corresponding to the helices C−D, E−F, and H (last 10 residues), respectively). These results indicated that the I-1 species consists of specific structured regions separated by unordered regions endowed with higher flexibility. A completely different picture emerged when the HFIP-MG_N intermediate was examined. The analysis of the peptic digest revealed that the exchange rate was similar for all protein regions. No individual region of the protein was specifically affected by the presence of HFIP.

DISCUSSION

The addition of HFIP to native myoglobin is known to disrupt the tertiary structure as documented by the complete loss of the aromatic side chain CD signals in the near-UV as well as the visible and near-UV absorption bands arising from the interaction of the heme with the protein matrix. Conversely, the amount of secondary structure is much less affected (i.e., 80% of secondary structure survives the fluoro alcohol induced conformational transition (*32*)). The biological importance of non-native protein conformations ranging from various denatured conformations to aggregated forms is continuously emphasized by recent research (*63*−*65*). This brought our attention to the fluorescence properties of the complex formed by HFIP-MG_N. Using the extrinsic fluorescent probe ANS we compared the data with those relative to other molten globule-like states obtained under different experimental conditions. The acid- and alcohol-destabilized forms of myoglobin are of great interest in studying protein folding. In fact, progressively destabilized forms may provide a representation of the folding path if one assumes that the most stable parts form first. The fluorescence studies have been integrated with amide HDX rate studies, a technique that provides detailed information on protein structure and dynamics. Although NMR is typically used to provide these data, its use requires high protein concentration. Mass spectrometry is not subject to this limitation for measuring deuterium incorporation into proteins, and therefore, provides information, which is not affected by concentration-dependent protein−protein interactions.

The fluorescence emission of the complexes indicate that the energy transfer via the Förster mechanism from tryptophans to ANS is much less efficient for the molten globule state obtained by adding 4% v/v HFIP to native myoglobin (i.e., HFIP-MG_N state) than the partly folded states formed by protein under different experimental conditions. The transfer efficiency is related to distance between the two fluorophores and to the mutual orientation between the emission dipole of the donor and the excitation dipole of the acceptor. Thus, an efficiency reduction can be ascribed to an increase of distance or to a change in the angle between the two dipole moments. The ANS polarization increase detected for HFIP-MG_N suggests that this state is more expanded than the other states formed under different experimental conditions, and therefore, supports the hypothesis that the distance between the two fluorophores is increased.

The results from the HDX experiments on the two intermediates, HFIP-MG_N and I-1, are consistent with the spectroscopic data showing that their conformations are sensibly different. In mild acidic conditions (I-1), myoglobin retains some tertiary structure (i.e., the structured subdomain formed by the A, G, and H helices). By contrast, most of the tertiary interactions are lost upon addition of HFIP to native myoglobin resulting in a less compact, although still globular conformational state. The experimental data indicate that the conformational state adopted in the presence of 4% v/v HFIP possesses the structural features typical of the so-called molten globule state (i.e., a native-like secondary structure and a fluctuating tertiary organization).

In conclusion, the picture that emerges is that the HFIP-MG_N is a compact intermediate state with no fixed structure. It has considerable conformational flexibility. In this respect, it may represent one of the first forms, if not the earliest, appearing on the folding path of apomyoglobin. In fact, even the less structured of the acid-destabilized forms of apomyoglobin (i.e., the E state) contains more specific sets of tertiary interactions than is generally imagined in the hypothetical folding path that emphasizes an overall hydrophobic collapse with few or no fixed tertiary contacts.

The mechanism by which HFIP induces formation of a compact destabilized protein conformation still remains unclear but is probably distinct from that of salts and polyols that are also known to stabilize partly folded states. Previous studies have shown that the alcohol induced denaturation of proteins can be interpreted in terms of two process: disruption of the native state and induction of an α-helical conformation. In many cases, the process results in the formation of extended helical rods in which the hydrophobic side chains are exposed, whereas the polar amide groups are shielded from the solvent (*21*, *26*, *27*). Here we show that the effects induced by HFIP on myoglobin conformation are dependent on both pH and preexisting protein state. When HFIP is added to native protein, the expanding force exerted by its nonpolar character destabilizes most of the fixed tertiary interactions, resulting in the formation of a true molten globule. When HFIP is added to acid unfolded apomyoglobin, the force inducing secondary structure becomes prevalent and increases the populations of those helices that have relatively good intrinsic helix propensities. This produces a species that binds ANS with energy transfer efficiency similar to that observed for I-1. However, in these conditions, it is unlikely that the A, G, and H helices interact with each other as a core.

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BI020447F